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Selection of synthetic receptors capable of sensing the difference in binding of D-Ala-D-Ala or D-Ala-D-Lac ligands by screening of a combinatorial CTV-based library

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Abstract—Screening of a combinatorial CTV-based artificial, synthetic receptor library **1** {1-13, 1-13, 1-13} for binding of a variety D-Ala-D-Ala and D-Ala-D-Lac containing ligands (**6**–**11**) was carried out in phosphate buffer (0.1 N, pH=7.0). After screening and Edman sequencing, synthetic receptors were found containing amino acid sequences, which are either characteristic for binding dye labeled D-Ala-D-Ala or D-Ala-D-Lac containing ligands. For example, receptors capable of binding D-Ala-D-Ala containing ligands **6**, **7**, **9** and **11** contained—almost in all cases—at least one basic amino acid residue—predominantly Lys—in their arms. This was really a striking difference with the arms of the receptors capable of binding D-Ala-D-Lac containing ligands **8** and **10**, which usually contained a significant number of polar amino acids (Gln and Ser), especially in ligand **8**, but hardly any basic amino acids. Use of different (fluorescent) dye labels showed that the label has a profound, albeit not decisive, influence on the binding by the receptor. A hit from the screening of the CTV-library with FITC-peptidoglycan (**6**) was selected for resynthesis and validation.

1. Introduction

The identification of artificial synthetic receptor molecules capable of binding specific peptide sequences has attracted considerable interest.[1] Many of these receptors have been designed, prepared and screened for their ability to bind resin attached peptides from combinatorial libraries.

Combinatorial solid phase synthesis offers an attractive strategy for the generation of libraries of synthetic receptor molecules capable of binding specific peptide sequences.[2]

Recently, we have prepared a 2197-member split-mix[3] combinatorial library of artificial tripodal receptors **1**{1-13, 1-13, 1-13} on the solid phase,[4] using cyclotriveratrylene (CTV)[5] as a scaffold (Scheme 1). Thus, every bead contains one unique CTV-based receptor with three peptide arms, one of them attached to the solid phase and different from the other two identical peptide arms. Selectivity and affinity for ligands might be enhanced, since the CTV-scaffold is clearly capable of aligning peptide arms.[6]

The CTV-based receptors were attached to Argogel®-NH_2 resin, therefore an 'on-bead screening assay'[3c,7] for binding of different peptide sequences by the CTV-based combinatorial library **1**{1-13, 1-13, 1-13} can be performed. Beads containing receptors capable of binding particular ligands are visually identified and removed for structural elucidation by Edman degradation.[8] In order to visualize the best CTV-receptor binders, fluorescence or staining of the library beads resulting from binding a specific peptide sequence or ligand in solution is an attractive approach. For this purpose labeling with a (fluorescent) dye of these peptides or ligands is required.

As a still very challenging example of screening for binding of specific peptide sequences by the CTV-based library **1**{1-13, 1-13, 1-13}, peptide sequences D-Ala-D-Ala and

Keywords: Synthetic receptors; CTV-based library; Screening; Fluorescent ligand; D-Ala-D-Ala-OH; D-Ala-D-Lac-OH.

Abbreviations: Boc, *tert*-butoxycarbonyl; BOP, benzotriazol-tris-(dimethylamino)phosphonium hexafluoro-phosphate; ^{t}Bu, *tert*-butyl; Cbz, benzyloxycarbonyl; CTV, 2,7,12-trihydroxy-3,8,13-trimethoxy-10,15-dihydro-5*H*-tribenzo[a,d,g]cyclononene; DCM, dichloromethane; DiPEA, *N*,*N*-diisopropyl-*N*-ethylamine; DMF, dimethylformamide; Ds, Dansyl; ES-MS, elctrospray ionization mass spectrometry; EtOAc, ethyl acetate; Et_2O, diethyl ether; FITC, fluorescein isothiocyanate; Fmoc, *N*-fluoren-9-ylmethoxy-carbonyl; NBD, 7-nitrobenz-2-oxa-1,3-diazol; NMP, *N*-methylpirrolidone; Rt, retention time; rt, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid.

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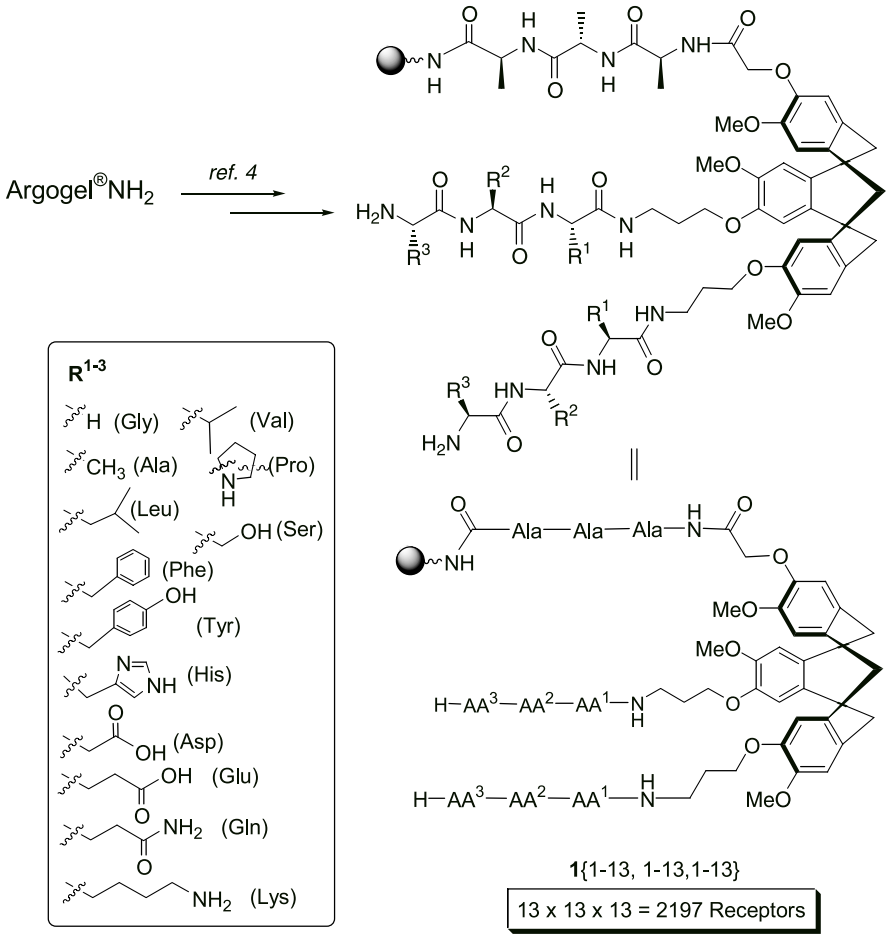


Scheme 1. Combinatorial synthesis of a 2197-member library of CTV-based tripodal receptors **1**{1-13, 1-13, 1-13}.[4]

D-Ala-D-Lac were chosen. Vancomycin and other closely related glycopeptide antibiotics[9] bind to the D-Ala-D-Ala end of the Gram-positive bacterial peptidoglycan cell wall precursor thereby interfering with its cross-linking, resulting in an incomplete bacterial cell wall, leading to osmotic lysis and bacteria death.[10] Unfortunately, the terminal D-alanine is substituted by D-lactate in vancomycin-resistant bacterial strains, resulting in a 1000-fold decrease in affinity and essentially useless antibiotic activity of vancomycin.[11] Thus, identification of synthetic receptors capable of binding with high affinity to the above mentioned sequences could have a potential application for overcoming vancomycin resistance problems. This is nevertheless an extremely difficult problem to address, since the number of interactions of the small D-Ala-D-Ala and D-Ala-D-Lac ligands with either 'synthetic' or 'natural' receptors (viz. vancomycin) is very limited. Despite this, Nature managed to come up with the natural 'receptor' vancomycin having a high affinity for D-Ala-D-Ala ($K_a \sim 10^6\ M^{-1}$).[12]

In this report a 2197-member library of CTV-based synthetic tripodal receptor **1**{1-13, 1-13, 1-13} attached to the solid phase was screened for binding of D-Ala-D-Ala and D-Ala-D-Lac containing ligands, in phosphate buffer (0.1 N, pH = 7.0). So far there are not many examples of screening for binding properties by synthetic receptors in aqueous systems.[2a–b,13] However, this was deemed absolutely essential for moving ultimately to biologically relevant systems and finding hits for these. We found that it is no longer difficult to find hits of synthetic receptors with good to excellent binding properties in organic solvents.[1c,f,h,2b–c] The challenge, however, is to discover molecules with good binding properties in an aqueous environment, since intermolecular interactions (hydrogen bond, electrostatic) between ligand and receptor are much weaker here than in apolar solvents.

Since D-Ala-D-Ala or D-Ala-D-Lac ligands are relatively small, the influence of the (fluorescent) dye was evaluated by varying it from fluorescein (FITC), dansyl (Ds), or 7-nitrobenz-2-oxa-1,3-diazol (NBD), to disperse red (DR). The best synthetic receptors were selected and subjected to Edman degradation[8] and the binding sequences were analyzed. Validation of 'hits' by resynthesis on the solid phase of the selected library member has been carried out. The resynthesized receptors have been tested in vitro against *Staphylococcus aureus* Newman.

One can philosophize how Nature did find such a good candidate for binding as vancomycin. However, it seems plausible that the 'natural' equivalent of combinatorial approaches, i.e. evolution led to the emergence of such a good binder for binding D-Ala-D-Ala. Similarly, we propose that ultimately combinatorial approaches will lead to new binders and possibly antibiotics. Here, we describe the screening of a CTV library as a step in this direction.

2. Results and discussion

In order to investigate the binding properties for D-Ala-D-Ala and D-Ala-D-Lac containing ligands by the fully deprotected recently described CTV-based receptor library **1**{1-13, 1-13, 1-13}[4], fluorescent FITC-peptidoglycan,[14] (**6**), NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Ala-OH (**7**), and Ds-Gly-D-Ala-D-Ala-OH (**9**), as well as red-colored ligand DR-C(O)–$(CH_2)_2$–C(O)-Gly-D-Ala-D-Ala-OH (**11**) were prepared corresponding to cell wall precursors of vancomycin sensitive bacteria. In addition, fluorescent NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Lac-OH (**8**), and Ds-Gly-D-Ala-D-Lac-OH (**10**) were synthesized as a small parts of cell wall precursors of vancomycin resistant bacteria. The (fluorescent) dye labels were chosen to study their possible influence on binding.

2.1. Synthesis of the (fluorescent) dye labeled D-Ala-D-Ala and D-Ala-D-Lac containing ligands (7–11)

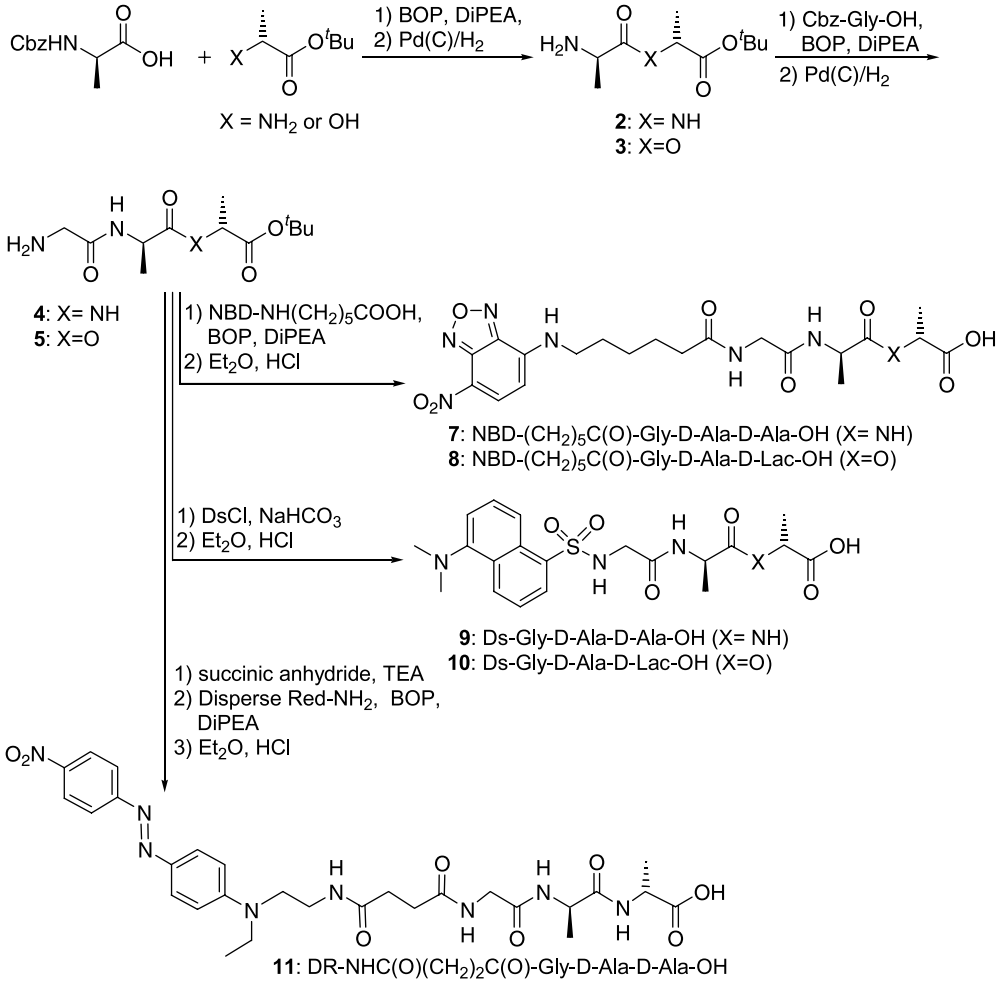
The D-Ala-D-Ala containing ligands (**7**, **9** and **11**) were prepared by attachment of the different fluorescent or dye labels to H-Gly-D-Ala-D-Ala-O^{t}Bu (**4**) (Scheme 2). The common intermediate **4** was obtained by BOP coupling[15] of commercially available Cbz-D-Ala-OH and H-D-Ala-O^{t}Bu and hydrogenolysis to the dipeptide H-D-Ala-D-Ala-O^{t}Bu (**2**) (92%), followed by BOP coupling of Cbz-Gly-OH to **2**, and again hydrogenolysis.

NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Ala-OH (**7**) was synthesized by BOP coupling of fluorescent 6-(7-NitroBenzo-2-oxa-1,3-Diazole) hexanoic acid to **4** and cleavage of the *t*-Bu-ester under acidic conditions. Treatment of **4** with a slight excess of dansyl chloride in the presence of $NaHCO_3$ and cleavage of the *t*-Bu-ester led to the dansylated D-Ala-D-Ala containing ligand (Ds-Gly-D-Ala-D-Ala-OH) **9**. Similar approaches were used, for the preparation of the NBD and Ds-labeled D-Ala-D-Lac containing ligands **8** and **10**. For these ligands H-Gly-D-Ala-D-Lac-O^{t}Bu (**5**) was used as a precursor. This precursor was synthesized analogously to **4**, but using H-D-Lac-O^{t}Bu instead of H-D-Ala-O^{t}Bu (Scheme 2).

Disperse red D-Ala-D-Ala containing ligand (**11**) was synthesized by treatment of **4** with succinic anhydride under basic conditions followed by BOP coupling of disperse red-NH_2[16] and final cleavage of *t*-Bu-ester.

2.2. Screening of library 1{1-13, 1-13, 1-13} for binding of D-Ala-D-Ala or D-Ala-D-Lac containing ligands

The fully deprotected CTV-based receptor library **1**{1-13, 1-13, 1-13} was screened for binding of the dye containing



Scheme 2. Synthesis of the (fluorescent) dye labeled D-Ala-D-Ala and D-Ala-D-Lac containing ligands (**7**–**11**).

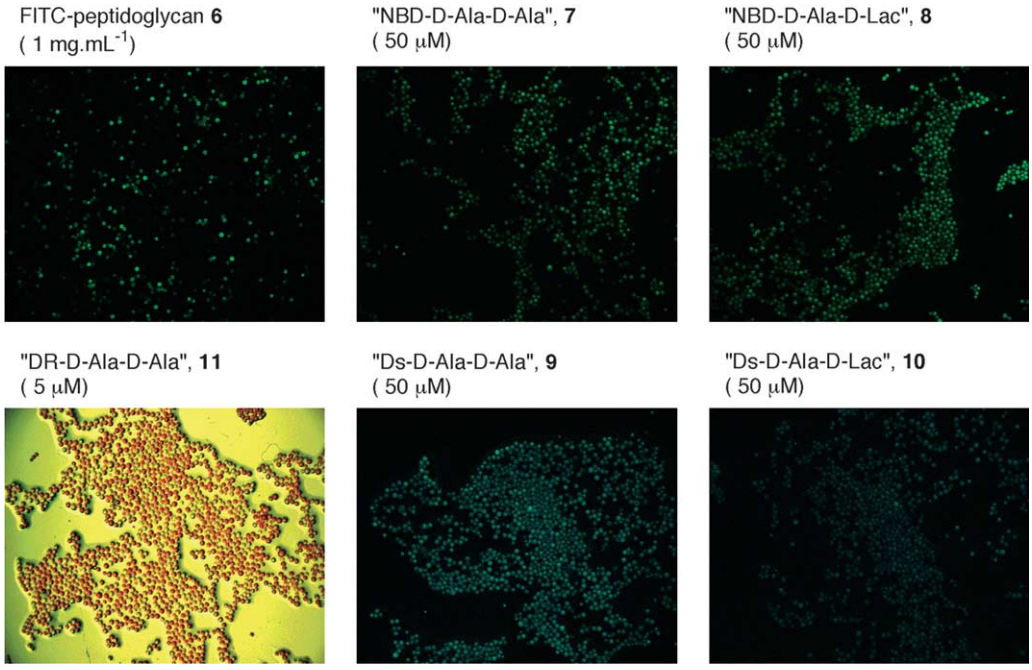


Figure 1. Screening for binding for D-Ala-D-Ala and D-Ala-D-Lac containing ligands (**6–11**) by the CTV-based receptor library **1**{1-13, 1-13, 1-13}.

D-Ala-D-Ala or D-Ala-D-Lac ligands (**6–11**) in phosphate buffer (0.1 N, pH = 7.0). Although this aqueous system is a biologically more meaningful environment, peptide recognition by the artificial receptors is more difficult than in the frequently used apolar organic systems due to the ability of water molecules to compete for hydrogen bonding by solvation and thus disruption of these non-covalent interactions between peptides and synthetic receptors. For the fluorescent dyes the overexposure mode of the Leica DC-100 digital camera system and image analysis was used for estimation of the relative fluorescence intensities in a semi-quantitative manner. Different ligand concentrations were used to determine the optimal selectivity, as judged by the number of highly fluorescent beads against either no or slightly fluorescent beads. For NBD- or Ds-labeled ligands (**7–10**), no selectivity of binding at higher concentrations than 50 μM was observed. At concentrations lower than 10 μM, fluorescent beads were barely visible. Good discrimination between fluorescent and non-fluorescent beads was possible at 50 μM, and therefore this concentration was used for screening experiments with fluorescent ligands (**7–10**). FITC-labeled peptidoglycan fragment ligand (**6**) was tested at concentrations of 10, 1 and 0.1 mg mL^{-1}, leading to the best discrimination when a concentration of 1 mg mL^{-1} was used. The best discrimination between red and colorless beads was obtained when 5 μM of the disperse-red labeled ligand **11** was used. In general, the observed fluorescence intensity was slightly higher for D-Ala-D-Ala containing ligands than for D-Ala-D-Lac containing ligands. This might be

Table 1. Identity of the peptide arms as obtained by Edman degradation of the selected solid phase attached CTV-based receptors

Bead	FITC-peptidoglycan **6** AA^3-AA^2-AA^1	" NBD-D-Ala-D-Ala" **7** AA^3-AA^2-AA^1	"NBD-D-Ala-D-Lac" **8** AA^3-AA^2-AA^1	" Ds-D-Ala-D-Ala" **9** AA^3-AA^2-AA^1	"Ds-D-Ala-D-Lac" **10** AA^3-AA^2-AA^1	" DR-D-Ala-D-Ala" **11** AA^3-AA^2-AA^1
1	Lys-Leu-Lys	Leu-Lys-Phe	Val-Phe-Phe	Ser-Lys-Ala	Ala-Leu-Val	Phe-Lys-His
2	Lys-Leu-Lys	Phe-Lys-Phe	Leu-Ala-Phe	Ser-Lys-Glu	Ala-Leu-Ala	Leu-Lys-Gly
3	Lys-Phe-Lys	Val-Lys-Phe	Phe-Ser-Phe	Ser-Lys-Gly	Val-Leu-Leu	Tyr-Lys-Ala
4	Lys-Lys-Lys	Leu-Val-Lys	Val-Gly-Phe	Gly-Lys-Ala	Val-Phe-Leu	Lys-Phe-Tyr
5	Ala-Ala-Lys	Leu-Phe-Lys	Phe-Phe-Gln	Gly-Lys-Val	Ala-Ala-Gln	Lys-Leu-Phe
6	Ala-Val-Lys	Phe-Val-Lys	Leu-Phe-Thr	Gly-Ala-Lys	Val-Ala-Gln	Gly-Gly-Lys
7	Phe-Val-Lys	Tyr-Leu-Lys	Gln-Phe-Val	Lys-Phe-Phe	Gly-Lys-Gly	Ala-Leu-His
8	Val-Pro-Lys	Lys-Phe-Leu	Gln-Ser-Leu	Lys-Leu-Leu	Leu-Phe-Gly	Val-Gly-Ala
9	Lys-Lys-Val	Leu-Gln-Phe	Ser-Leu-Gln	Ser-Ala-Gly	Lys-Phe-Val	Ala-Phe-Leu
10			Leu-Pro-Val	Ser-Gly-Ala	Leu-Lys-Gln	
NF	Ser-Ala-Glu	Gln-Asp-Glu	Pro-Asp-Glu	Phe-Val-Asp	Pro-His-Asp	Phe-Asp-Ser

NF, non-fluorescent bead.

a qualitative indication of stronger binding of the D-Ala-D-Ala sequence. A higher fluorescence intensity was generally observed when NBD-label ligands were used instead of the Ds-label. The best selectivity was found when FITC-peptidoglycan (**6**) was used as ligand (Fig. 1).

For every independent screening nine or ten of the most intensively fluorescent beads were selected and sequenced by Edman degradation.[8] Edman sequencing was carried out in all the cases for a fourth cycle, which always showed no amino acid for all the beads, thus confirming the *tripeptide*-identity of the library members. In addition, a non-fluorescent bead was selected and sequenced as a negative control.

Table 1 shows the identity of the peptide arms of the selected solid phase attached CTV-based receptors. In Figure 2, the relative abundance of amino acids at each residue position of the receptor is shown. The general trends are very clear and can be summarized as follows. First, the negative controls, i.e. the selected non-fluorescent or non-colored beads, contained amino acids especially Glu and Asp in their receptors, which were not found in the fluorescent or colored beads. Second, the receptors capable of binding of D-Ala-D-Ala containing ligands **6**, **7**, **9** and **11** contained—almost in all cases—at least one basic amino acid residue (indicated in blue in Table 1)—predominantly Lys—in their arms. This was really a striking difference with the arms of the receptors capable of binding D-Ala-D-Lac containing ligands **8** and **10**, which usually contained a significant number of polar amino acids (Gln and Ser), especially in binding ligand **8**, but hardly any basic amino acids. Although there was a significant influence of the label (vide infra) on binding of the ligand to the synthetic receptors, fortunately, the labels did not conceal the differences in binding of D-Ala-D-Ala or D-Ala-D-Lac ligands, i.e. they did not obscure the selectivity of binding. The differences in the sequences of the receptors in binding of D-Ala-D-Ala or D-Ala-D-Lac remained manifest irrespective of the (fluorescent) dye label. Nevertheless, there were appreciable differences in sequences of receptors capable of binding the D-Ala-D-Ala with the various labels. For example, the synthetic receptors capable of binding

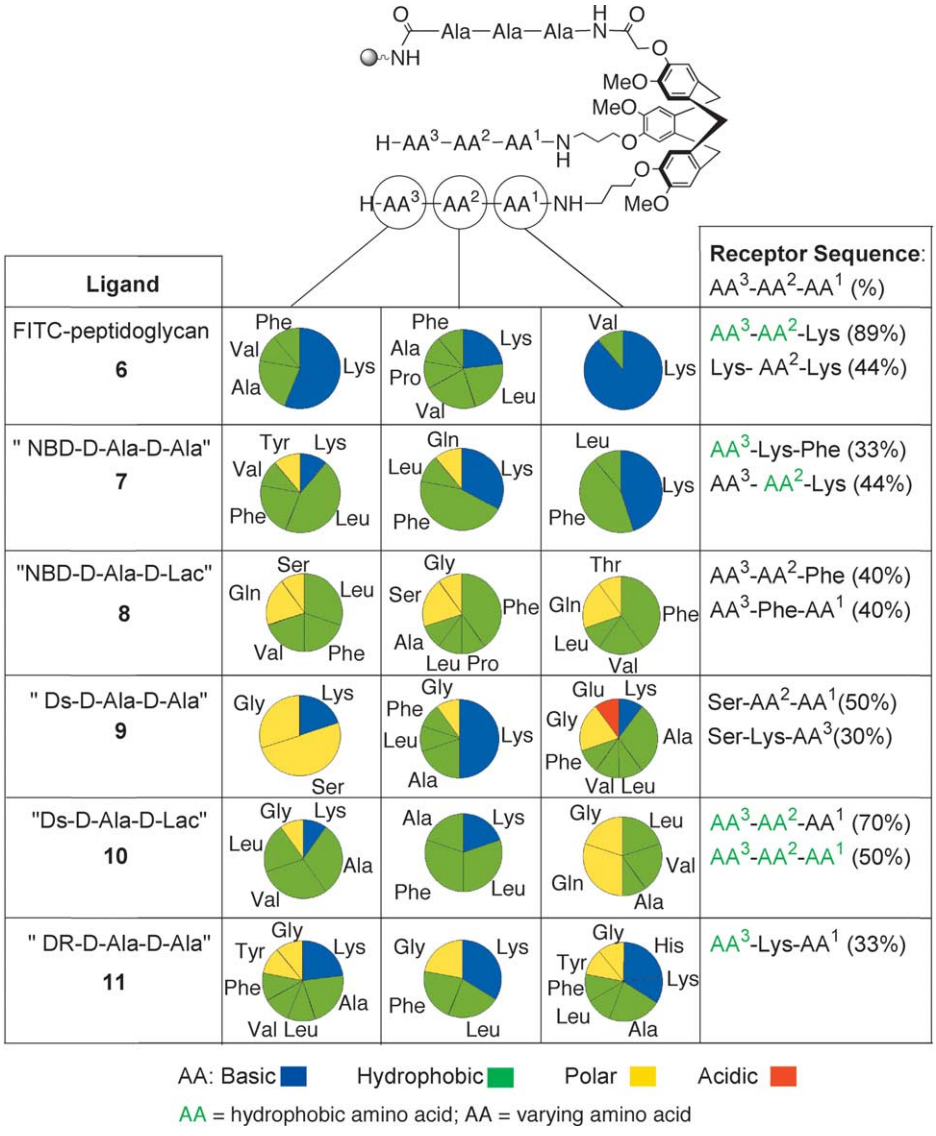


Figure 2. Relative abundance of amino acids at each residue position of the CTV-based receptor.

'NBD-D-Ala-D-Ala' (**7**) contained—in addition to Lys—virtually only hydrophobic amino acids (Leu, Phe, Val), whereas the synthetic receptors capable of binding 'Ds-D-Ala-D-Ala' (**9**) contained—in addition to Lys—a number of more polar Ser residues as well as the smaller hydrophobic amino acids such as Ala. The behavior of synthetic receptors, which bound 'DR-D-Ala-D-Ala' (**11**) was somewhat intermediate: Lys residues, large hydrophobic amino acids (Leu, Phe) but also the smaller hydrophobic amino acid Ala was found, but no polar amino acids like Ser. Thus, we found synthetic receptors selective for binding either the D-Ala-D-Ala or the D-Ala-D-Lac sequence. As such these compounds can be considered as 'sensors' for these particular peptide sequences and synthetic receptors acting as sensors for other peptide ligands might be accessible using this combinatorial approach. Possibly, by simply screening this library with another peptide ligand one might even uncover synthetic receptors acting as sensors for other peptides. The binding behavior of the synthetic receptors—as reflected in the amino acid sequences—was influenced by the character of the (fluorescent) dye label, but the selectivity remained.

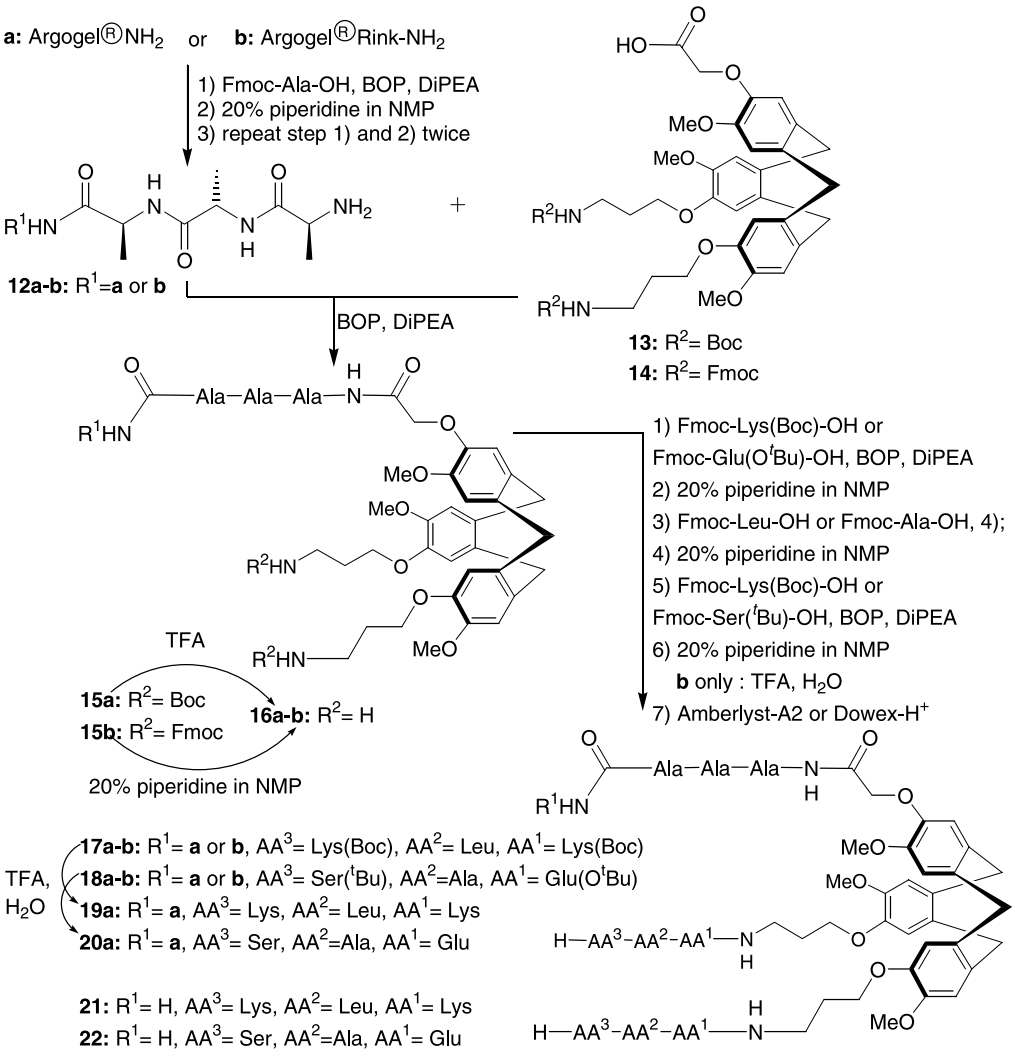
In order to reduce the influence of the label, which is required for screening, different labels which have a minimal influence on binding, are called for. Alternatively, the relative influence of the label on binding by a particular synthetic receptor can be diminished by screening synthetic receptors capable of binding larger ligands as compared to the size of the label. The found selectivities for binding D-Ala-D-Ala vs. binding D-Ala-D-Lac are quite remarkable considering the fact that the only difference between these ligands is present in the linkage connecting the two residues: an amide 'N–H' in D-Ala-D-Ala and an ester '–O' in D-Ala-D-Lac.

It is revealing to look at the relative abundance of the various amino acids at each residue position of the receptor shown in Figure 2. In 89% of the sequenced synthetic receptors capable of binding fluorescein labeled D-Ala-D-Ala (FITC-peptidoglycan, **6**), Lys is found at the first position (AA^1), closest to the CTV-scaffold. Lys in this position is also found in 44% of the receptors capable of binding 'NBD-D-Ala-D-Ala' (**7**). Although in 44% of the receptors binding FITC-peptidoglycan (**6**), Lys is found at the third position (AA^3), Lys can also be present at the second position (AA^2) in 33% of the receptors binding 'NBD-D-Ala-D-Ala' (**7**), in 30% of the receptors binding 'Ds-D-Ala-D-Ala' (**9**) and in 33% of the receptors binding 'DR-D-Ala-D-Ala' (**11**). As was discussed above, the absence of Lys residues in synthetic receptors capable of binding D-Ala-D-Lac in ligands **8** and **10** is remarkable.

Likewise, the combination of the basic Lys residue with hydrophobic amino acids indicated in green in the receptor sequences, when a varying hydrophobic amino acid is found. Especially with the NBD-ligands **7** and **8**, Phe was found in 33% of the receptors binding D-Ala-D-Ala (in ligand **7**) and in 80% (40% AA^1 and 40% AA^2) of the receptors binding D-Ala-D-Lac (in ligand **8**). The presence of this particular amino acid may point to a special contribution of the NBD label on binding, since this amino acid was not in majority present in the receptors binding the other ligands. Nevertheless the presence of hydrophobic amino acids was very striking in the receptors capable of binding D-Ala-D-Lac, i.e. **8** and **10**. In the latter case even 50% of the sequenced receptors contained hydrophobic amino acids at each of the three amino acid positions and a good 70% had at least two hydrophic amino acids, i.e. at the third (AA^3) and second (AA^2) position of the synthetic receptors. Finally, the presence of Ser in the third position in 50% of the synthetic receptors binding 'Ds-D-Ala-D-Ala' (**9**) was also surprising and in 30% of the receptors the consensus sequence Ser-Lys-AA^1 was found. This cannot be solely ascribed to an effect of the Ds-label, as the corresponding Ds-containing D-Ala-D-Lac ligand did not bind to receptors containing Ser residues.

2.3. Validation by resynthesis

Validation of 'hits' by resynthesis of the selected synthetic receptors and reproducing the properties for which the library (**1**{1-13, 1-13, 1-13}) was screened is essential in any combinatorial approach towards finding compounds with desired—in this case binding—properties. A hit from the screening of the CTV-library with FITC-peptidoglycan (**6**) was selected, which was found twice (Table 1, beads 1 and 2, screening with **6**), corresponding to the consensus sequence Lys-AA^2-Lys found in 44% of the sequenced beads. In addition the 'negative control', i.e. a sequenced non-fluorescent bead giving the CTV-based synthetic receptor sequence Ser-Ala-Glu, (AA^3-AA^2-AA^1) was synthesized on Argogel®-NH_2 resin (**a**) as well as on the Argogel®-Rink resin (**b**) (Scheme 3). Resynthesis of the CTV-based receptors **19a** and **20a**, covalently attached to Argogel®-NH_2 resin, and **17b** and **18b** on the Argogel®-Rink resin, was performed analogously to the preparation of the CTV-based receptor library **1**{1-13, 1-13, 1-13}[4] except for the use of the compatible $(Fmoc)_2$-CTV-OH scaffold **14** on the acid labile Argogel®-Rink resin, instead of the $(Boc)_2$-CTV-OH scaffold **13**. The different peptide-binding arm was built up by three subsequent BOP couplings/Fmoc-deprotection of Fmoc-L-Ala-OH on both, Argogel®-NH_2 and Argogel®-Rink-NH_2 resin to obtain **12a** and **12b**, respectively (Scheme 3). Then, either the Boc-protected CTV-scaffold (**13**) or the Fmoc-protected CTV-scaffold (**14**) was coupled to the first peptide-binding arm to give **15a** or **15b**. Cleavage of the Boc groups under acidic conditions from **15a** led to **16a**, while Fmoc-cleavage under standard conditions from **15b** led to **16b**. The construction of the two identical peptide arms was performed as depicted in Scheme 3. After three coupling/Fmoc-deprotection cycles involving subsequently Fmoc-L-Lys(Boc)-OH, Fmoc-L-Leu-OH and Fmoc-L-Lys(Boc)-OH, resin bound synthetic receptors **17a** and **17b** were obtained. The same procedure involving the use of subsequently Fmoc-L-Glu(O^{t}Bu)-OH, Fmoc-L-Ala-OH and Fmoc-L-Ser(^{t}Bu)-OH led to resin bound synthetic receptors **18a** and **18b**. Treatment of **17a** and **18a** with TFA led to the—still resin bound—deprotected synthetic receptors, **19a** and **20a**, respectively (Scheme 3). In contrast, treatment with TFA of **17b** or **18b** led to deprotection and cleavage of the receptors from the Argogel®-Rink-resin to afford the trifluoroacetate salts of [Lys-Leu-Lys]-containing or [Ser-Ala-Glu]-containing synthetic receptors **21** and **22**, respectively (Scheme 3). ES-MS showed the identity of the trifluoroacetate salts receptors **21** and **22**. The purity of them was 94% purity in both cases, as shown by HPLC. The



Scheme 3. Resynthesis of resin bound (**19a** and **20a**) and cleaved synthetic receptors (**21** and **22**).

overall yields (76 and 69%, respectively) were very good considering the eight steps performed for their solid-phase synthesis (corresponding to 97 and 95% average yield per step). Finally, desalting using either Amberlyst–A21 or Dowex-H^+ ion exchange resins led to receptors **21** and **22**, respectively (Scheme 3).

Binding experiments of the resynthesized CTV-based

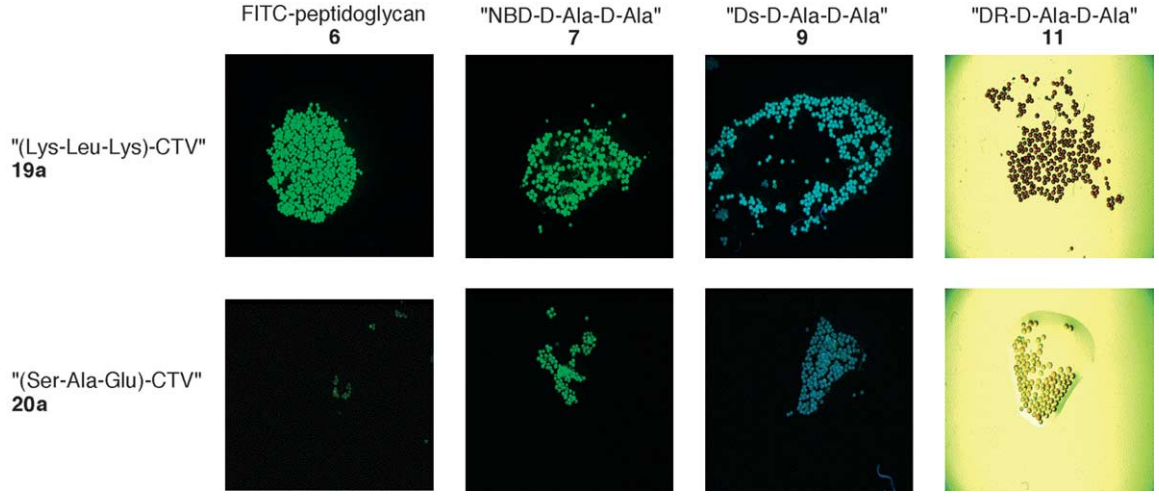


Figure 3. Screening for binding for D-Ala-D-Ala and D-Ala-D-Lac containing ligands (**6**, **7**, **9**, and **11**) by the resynthesized CTV-based receptor **19a** and **20a**.

receptors **19a** and **20a** was carried out by incubation with a 1 mg mL^{-1} suspension of FITC-peptidoglycan (**6**). Homogeneous fluorescent beads were observed for receptor **19a**, clearly showing affinity for FITC-peptidoglycan while no fluorescence was observed for the control receptor **20a** (Fig. 3). These findings independently confirmed the identity of the receptors as obtained by the split-mix procedure.

In order to investigate whether the affinity of the resynthesized CTV-based receptor **19a** was due to either their interaction with D-Ala-D-Ala or with a different part from the FITC-peptidoglycan ligand (**6**), the binding of resin bound receptor **19a** for NBD, Dansyl- and disperse-red-D-Ala-D-Ala containing ligands **7**, **9**, and **11** was tested. For that purpose the [Lys-Leu-Lys]-containing CTV receptor **19a** and the 'control' [Ser-Ala-Glu]-containing CTV-based receptor **20a** were incubated with a 50 μM solution of ligands **7** or **9** or a 5 μM solution of ligand **11**. Homogeneous fluorescent beads were observed in all cases involving synthetic receptor **19a**, while no fluorescent beads were observed with control synthetic receptor **20a** (Fig. 3). Thus, binding is independent from the (fluorescent) dye label and really seems to involve recognition of the D-Ala-D-Ala sequence.

In view of these results, it was attempted to inhibit bacterial growth by the resynthesized [Lys-Leu-Lys]-containing CTV-receptor **21**. [Ser-Ala-Glu]-containing CTV-receptor **22** was also included in the experiments. A range of concentrations from 0 to 100 μM of **21** or **22** were used to determine a possible minimal inhibitory concentration (MIC) against *Staphylococcus aureus* Newman. Vancomycin was used as a positive control. Unfortunately, no inhibition of bacterial growth was observed for the CTV-based receptor **21** indicating no cell wall disruption and therefore no bacterial death. Negative control synthetic receptor **22** was also inactive. Although a possible explanation for the absence of antibacterial activity by synthetic receptor **21** might be too low a binding affinity for the D-Ala-D-Ala sequence, an alternative explanation is proteolytic degradation of the peptide arms of synthetic receptor **21**.

3. Conclusions

Thus by screening a CTV-based library containing tripeptide arms, one attached to the solid phase and two identical varying arms, we were able to find synthetic receptors containing amino acid sequences, which are either characteristic for binding dye labeled D-Ala-D-Ala or D-Ala-D-Lac containing ligands. This opens up possibilities for finding by combinatorial approaches synthetic receptors, which can act as 'sensors' for particular peptides. By attachment of fluorescent groups to these sensors or by immobilizing them to suitable surfaces one might be able to devise read-out systems for monitoring the extent of binding of peptide or other ligands. Selectivity of binding by synthetic receptors as sensors might be conveniently determined by exposing them to for example to a library of peptides, followed by measuring the relative binding affinity by mass spectrometry.

Use of different (fluorescent) dye labels showed that the label has a profound, albeit not decisive, influence on the binding by the receptor. In view of the facts that D-Ala-D-Ala as well as D-Ala-D-Lac are only small ligands, with very limited of interactions and that the only difference between these ligands is present in the linkage connecting the two residues: an amide 'N–H' in D-Ala-D-Ala and an ester '–O' in D-Ala-D-Lac, underlines the significance of the findings. Nevertheless, a resynthetisized receptor based on the sequences found in the library screening was inactive against Gram-positive bacteria. If degradation of the synthetic receptor by proteases is largely responsible for this observation, then synthetic receptors containing D-amino acids might be more promising, which is under study now.

4. Experimental

4.1. General

All reagents were purchased from commercial sources and used without further purification. Argogel®-NH_2 (0.40 mmol g^{-1}, average bead diameter 178 μm), Argogel®-Rink-NH-Fmoc (0.33 mmol g^{-1}, average bead diameter 164 μm) resins were purchased from Argonaut Technologies, Inc., Amberlys-A21 ion exchange resin (4.8 mmol g^{-1}) from Aldrich, and Dowex 50×8 (H^+ form, 20–50 mesh) from Fluka. The Argogel®-NH_2 resin was dried over P_2O_5. The protected amino acids were purchased from Alexis Corporations (Läufelfingen, Switzerland) and Advanced Chemtech Europe (UK). All reactions on the solid phase were performed in standard glassware or poly(ethylene) glycol (PE) syringes with PE frits. Peptide grade solvents, dried on molecular sieves were used for reactions and resin washing. Kaiser and bromophenol blue (BPB) tests were used for detection of primary amines on the solid phase.[17,18] Analytical thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60 F_{254} (0.25 mm) plates. Spots were visualised with UV light, ninhydrine, or Cl_2-TDM.[19] Column chromatography was carried out using Merck Kieselgel 60 (40–63 μm). ^1H NMR and ^{13}C NMR were obtained on a Varian 300 MHz spectrometer. Chemical shifts are given in ppm with respect to internal TMS for ^1H NMR. ^{13}C NMR spectra were recorded using the attached proton test (APT) pulse sequence. ES-MS experiments were performed on a Shimadzu LCMS QP8000 system. HPLC analyses were performed on a Shimadzu-10Avp (Class VP) using PL-ELS-1000 detector and UV detector operating at 220 and 254 nm. An Adsorbophere XL C18, 300 Å (4.6 mm× 250 mm, 5 μm) was used; flow rate 1 mL min^{-1}; two mobile phases (mobile phases A, 99.9% water, 0.1% TFA; mobile phase B, 5% water, 95% acetonitrile, 0.085% TFA) with a standard protocol, 0% B for 5 min to 100% B in 20 min, 100% B for 5 min to 0% B in 5 min and reequilibrated at 0% B for 5 min.

4.1.1. H-D-Ala-D-Ala-O^{t}Bu (2). To a solution of Cbz-D-Ala-OH (1.16 g, 5 mmol) and HCl·H-D-Ala-O^{t}Bu (0.91 g, 5 mmol) in DCM (30 mL), BOP (2.21 g, 5 mmol) and DiPEA (2.61 mL, 15 mmol) were added. After stirring at rt for 3 h, the reaction was evaporated to dryness in vacuo and

the residue was dissolved in EtOAc (60 mL). The solution was washed with Na_2CO_3 (1 M), $KHSO_4$ (1 M), H_2O, and brine. After drying (Na_2SO_4) and evaporating the solvent, Cbz-D-Ala-D-Ala-O^{t}Bu (1.61 g, 92%) was obtained as white solid and used without further purification. R_f=0.56 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.33 (s, 5H, Ph), 6.42 (bs, 1H, NH(CO)), 5.29 (bs, 1H, NH(CO)O), 5.10 (s, 2H, CH_2Ph), 4.40, 4.22 (2m, 2H, CHα), 1.44 (s, 9H, C(CH_3)$_3$), 1.37, 1.34 (2d, 6H, $CH_3\beta$, J=6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 171.8, 171.5 (COO, NH(CO)), 155.8 (NH(CO)O), 136.2 (Ar-*C*), 128.5, 128.1, 128.0 (Ar-*H*), 82.1 (*C*(CH_3)$_3$), 66.9 (*C*H_2Ph), 50.4, 48.6 (Cα), 27.9 (C(*C*H_3)$_3$), 18.4 ($CH_3\beta$).

To a cooled solution (ice bath) of Cbz-D-Ala-D-Ala-O^{t}Bu (1.40 g, 4 mmol) in methanol (200 mL), Pd on charcoal (10%) (20% w/w) was added and the suspension was stirred under H_2 pressure from a balloon at rt for 2 h. The reaction was filtered and the solvent was evaporated to give **2** (0.86 g, 99%) as a colorless syrup. R_f=0.14 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.67 (bs, 1H, NH(CO)), 4.40, 3.46 (2m, 2H, CHα), 1.43 (s, 11H, NH_2, C(CH_3)$_3$), 1.34, 1.30 (2d, 6H, $CH_3\beta$, J=6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 175.2, 172.4 (COO, NH(CO)), 81.7 (*C*(CH_3)$_3$), 50.6, 48.1 (Cα), 27.9 (C(*C*H_3)$_3$), 21.7, 18.4 ($CH_3\beta$).

4.1.2. H-D-Ala-D-Lac-O^{t}Bu (3). A solution of Cbz-D-Ala-OH (1.16 g, 5 mmol), H-D-Lac-O^{t}Bu (0.73 g, 5 mmol) and BOP (2.21 g, 5 mmol) in DCM (30 mL) was cooled to −20 °C and DiPEA (2.61 mL, 15 mmol) was added. After stirring for 3 h, the reaction was evaporated to dryness in vacuo and the residue was dissolved in EtOAc (60 mL). The solution was washed with Na_2CO_3 (1 M), $KHSO_4$ (1 M), H_2O, and brine. After drying (Na_2SO_4) and evaporating the solvent, the crude **3** was purified by column chromatography using DCM/methanol, 30:1 to give Cbz-D-Ala-D-Lac-O^{t}Bu (1.55 g, 88%) as a colorless syrup. R_f=0.52 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.34–7.29 (s, 5H, Ph), 5.26 (bs, 1H, NH(CO)O), 5.11 (d, 1H, CH_{2a}Ph, J=12.2 Hz), 5.06 (d, 1H, CH_{2b}Ph), 4.97 (m, 1H, CHα-Lac), 4.42 (m, 1H, CHα-Ala), 1.51, 1.45 (2d, 6H, $CH_3\beta$, J=6.9 Hz), 1.43 (s, 9H, C(CH_3)$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 172.5, 169.4 (2COO), 155.6 (NH(CO)O), 136.2 (Ar-*C*), 128.5, 128.1, 128.0 (Ar-*H*), 82.2 (*C*(CH_3)$_3$), 69.7 (Cα-Lac), 66.9 (*C*H_2Ph), 49.4 (Cα-Ala), 27.9 (C(*C*H_3)$_3$), 18.6, 16.8 ($CH_3\beta$)β). Hydrogenolysis of Cbz-D-Ala-D-Lac-O^{t}Bu (1.40 g, 4 mmol) was performed following the same procedure as was used for Cbz-D-Ala-D-Ala-O^{t}Bu. **3** (0.76 g, 87%) was obtained as a colorless syrup. R_f=0.33 (DCM/methanol, 5:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 4.94 (m, 1H, CHα-Lac), 4.11 (m, 1H, CHα-Ala), 2.30 (bs, 2H, NH_2), 1.45 (d, 3H, $CH_3\beta$-Lac, J=7.7 Hz), 1.43 (s, 9H, C(CH_3)$_3$), 1.34 (d, 3H, $CH_3\beta$-Ala, J=6.9 Hz).

4.1.3. H-Gly-D-Ala-D-Ala-O^{t}Bu (4). Coupling (see preparation of **2**) of Cbz-Gly-OH (0.79 g, 3.8 mmol) to **2** (0.82 g, 3.8 mmol) gave Cbz-Gly-D-Ala-D-Ala-O^{t}Bu (1.38 g, 89%) as a white foam without further purification. R_f=0.76 (DCM/methanol, 5:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.32 (s, 5H, Ph), 6.82 (d, 1H, NH(CO), J=7.1 Hz), 6.73 (d, 1H, NH(CO), J=7.4 Hz), 5.60 (bs, 1H, NH(CO)O), 5.09 (s, 2H, CH_2Ph), 4.53, 4.38 (2m, 2H, CHα), 3.87 (d, 2H, CH_2-Gly, J=5.2 Hz), 1.43 (s, 9H, C(CH_3)$_3$), 1.35, 1.33 (2d, 6H, $CH_3\beta$, J=6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 171.8, 171.3, 168.6 (COO, 2NH(CO)), 156.6 (NH(CO)O), 136.1 (Ar-*C*), 128.5, 128.2, 128.1 (Ar-*H*), 82.1 (*C*(CH_3)$_3$), 67.2 (*C*H_2Ph), 48.8 (Cα), 44.4 (Cα-Gly), 27.9 (C(*C*H_3)$_3$), 18.7, 18.3 ($CH_3\beta$). ES-MS (m/z)=430.35 (100%, [M+Na]$^+$), 408.30 (9%, [M+H]$^+$), 352.25 (42%, [M−^{t}Bu+H]$^+$).

Hydrogenolysis of Cbz-Gly-D-Ala-D-Ala-O^{t}Bu (1.30 g, 3.19 mmol) gave **4** (0.85 g, 98%) as a colorless syrup. R_f=0.06 (DCM/methanol, 5:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.71 (d, 1H, NH(CO), J=7.7 Hz), 7.04 (d, 1H, NH(CO), J=6.9 Hz), 4.51, 4.33 (2m, 2H, CHα), 3.29 (s, 2H, CH_2-Gly), 1.39 (s, 11H, NH_2, C(CH_3)$_3$), 1.33 (d, 3H, $CH_3\beta$, J=6.9 Hz), 1.28 (d, 3H, $CH_3\beta$, J=7.1 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 172.7, 171.8, 171.7 (COO, 2NH(CO)), 81.7 (*C*(CH_3)$_3$), 48.6, 48.2 (Cα), 44.6 (Cα-Gly), 27.8 (C(*C*H_3)$_3$), 18.4, 18.1 ($CH_3\beta$).

4.1.4. H-Gly-D-Ala-D-Lac-O^{t}Bu (5). Overnight coupling (see preparation of **2**) of Cbz-Gly-OH (0.67 g, 3.2 mmol) to **3** (0.70 g, 3.2 mmol) gave Cbz-Gly-D-Ala-D-Lac-O^{t}Bu (1.28 g, 98%) as a colorless syrup after column chromatography using a gradient DCM/methanol from 40:1 to 20:1. R_f=0.77 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.33 (s, 5H, Ph), 6.51 (d, 1H, NH(CO), J=7.4 Hz), 5.40 (bs, 1H, NH(CO)O), 5.12 (s, 1H, CH_2Ph), 4.95, 4.62 (2m, 2H, CHα), 3.88 (s, 2H, CH_2-Gly), 1.45, (2d, 6H, $CH_3\beta$, J=7.1 Hz), 1.43 (s, 9H, C(CH_3)$_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 172.2, 169.3, 168.5 (2COO, NH(CO)), 156.6 (NH(CO)O), 136.1 (Ar-*C*), 128.5, 128.2, 128.1 (Ar-*H*), 82.3 (*C*(CH_3)$_3$), 69.8 (Cα-Lac), 67.2 (*C*H_2Ph), 47.9 (Cα-Ala), 44.4 (Cα-Gly), 27.9 (C(*C*H_3)$_3$), 18.3, 16.8 ($CH_3\beta$). Hydrogenolysis of Cbz-Gly-D-Ala-D-Lac-O^{t}Bu (1.20 g, 2.94 mmol) gave **5** (0.52 g, 64%) as a colorless syrup. R_f=0.16 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$ with a few drops of CD_3OD, 300 MHz) δ: 4.88, 4.47 (2m, 2H, CHα), 3.97 (d, 1H, CH_{2a}-Gly, J_{gem}=14.0 Hz, $J_{a,NH}$=6.9 Hz), 3.92 (d, 1H, CH_{2b}-Gly, $J_{b,NH}$=6.9 Hz), 1.39 (d, 6H, $CH_3\beta$, J=7.1 Hz), 1.39 (s, 9H, C(CH_3)$_3$).

4.2. Synthesis of D-Ala-D-Ala and D-Ala-D-Lac containing ligands (7–11)

4.2.1. NBD-(CH_2)$_5$-C(O)-Gly-D-Ala-D-Ala-OH (7). To a suspension of **4** (0.27 g, 1 mmol), 6-(7-nitrobenzo-2-oxa-1,3-diazole)-hexanoic acid (0.29 g, 1 mmol) and BOP (0.44, 1 mmol) in DCM (6 mL), DiPEA (0.35 mL, 2 mmol) was added. After stirring the orange solution at rt for 3 h, the reaction mixture was evaporated to dryness in vacuo and the residue was dissolved in EtOAc (6 mL). The solution was washed with $KHSO_4$ (1 M), H_2O, and brine. After drying (Na_2SO_4) and evaporating the solvent, the crude product was purified by column chromatography using a gradient of DCM/methanol from 30:1 to 10:1 and NBD-(CH_2)$_5$-C(O)-Gly-D-Ala-D-Ala-O^{t}Bu (0.45 g, 82%) was obtained as an orange solid. R_f=0.49 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.45 (d, 1H, H-6-NBD, J=8.8 Hz), 7.22 (bs, 1H, NH), 6.93 (d, 1H, NH(CO)-Ala, J=7.1 Hz), 6.65 (d, 1H, NH(CO)-Ala, J=7.1 Hz), 6.43 (t, 1H, NH(CO)-Gly, J=5.1 Hz), 6.14 (d, 1H, H-5-NBD), 4.52, 4.40 (2m, 2H, CHα), 4.00 (dd, 1H, CH_{2a}-Gly, J_{gem}=

16.7 Hz, $J_{a,NH}$=5.5 Hz), 3.92 (dd, 1H, CH_{2b}-Gly, $J_{b,NH}$=5.5 Hz), 3.52 (m, 2H, CH_2NH-NBD), 2.29 (t, 2H, CH_2C(O)-NBD, J=6.7 Hz), 1.83–1.48 (m, 6H, CH_2-NBD), 1.41 (s, 9H, $C(CH_3)_3$), 1.38, 1.34 (2d, 6H, $CH_3\beta$, J=6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 173.4, 171.7, 171.5, 168.5 (COO, NH(CO)), 144.3 (C-NBD), 136.7 (C-6, 5-NBD), 82.3 ($C(CH_3)_3$), 53.4 (Cα-Gly), 48.9 (Cα-Ala), 43.0, 35.7 (CH_2-NBD), 27.9 ($C(CH_3)_3$), 27.9, 26.2, 24.6 (CH_2-NBD), 18.8, 18.3 ($CH_3\beta$). ES-MS (m/z)=572.40 (100%, $[M+Na]^+$), 550.25 (45%, $[M+H]^+$), 4.94.35 (96%, $[M-^tBu+H]^+$).

Treatment overnight of NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Ala-O^{t}Bu (0.30 g, 0.54 mmol) with DCM/saturated HCl(g) in ether (1:1) (20 mL) led to an orange precipitate. Filtration and washing of the precipitate with cold ether gave to **7** (0.19 g, 70%) as an orange solid. R_f=0.04 (DCM/methanol, 5:1). ^{1}H NMR (DMSO, 300 MHz) δ: 8.51 (d, 1H, H-6-NBD, J=9.1 Hz), 8.33 (d, 1H, NH(CO), J=6.9 Hz), 8.06 (t, 1H, NH, J=6.0 Hz), 7.98 (d, 1H, NH(CO), J=7.4 Hz), 6.42 (d, 1H, H-5-NBD), 4.34–4.19 (m, 2H, CHα), 3.67 (d, 2H, CH_2-Gly, J=5.5 Hz), 3.35 (bs, 2H, CH_2NH-NBD), 2.14 (m, 2H, CH_2C(O)-NBD), 1.66, 1.54, 1.35 (3m, 6H, CH_2-NBD), 1.27, 1.19 (2d, 6H, $CH_3\beta$, J=7.1 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.4, 173.1, 172.4, 169.3 (COOH, NH(CO)), 144.2 (C-NBD), 137.0 (C-6,5-NBD), 58.0 (Cα-Gly), 48.0, 47.9 (Cα-Ala), 42.4, 35.3, 30.3, 26.0, 24.6 (CH_2-NBD), 17.7, 16.9 ($CH_3\beta$). ES-MS (m/z)=516.30 (40%, $[M+Na]^+$), 494.15 (100%, $[M+H]^+$). HPLC (Rt=17.75 min, 99% purity).

4.2.2. NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Lac-OH (8). The same procedure as was used for the preparation of **7** was followed. **5** (0.14 g, 0.5 mmol) gave NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Lac-O^{t}Bu (0.22 g, 80%) as an orange solid. R_f=0.59 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.46 (d, 1H, H-6-NBD, J=8.5 Hz), 6.87, 6.60, 6.30 (3bs, 3H, NH), 6.14 (d, 1H, H-5-NBD), 4.66–4.53 (m, 2H, CHα), 3.97 (s, 2H, CH_2-Gly), 3.50 (m, 2H, CH_2NH-NBD), 2.29 (t, 2H, CH_2C(O)-NBD, J=6.9 Hz), 1.86–1.70 (m, 4H, CH_2-NBD), 1.56–1.49 (m, 2H, CH_2NBD), 1.49, 1.46 (2d, 6H, $CH_3\beta$, J=7.1 Hz), 1.42 (s, 9H, $C(CH_3)_3$). ES-MS (m/z)=573.35 (100%, $[M+Na]^+$), 517.30 (45%, $[M-^tBu+Na]^+$), 495.13 (30%, $[M-^tBu+H]^+$).

Cleavage of the ^{t}Bu ester of NBD-$(CH_2)_5$-C(O)-Gly-D-Ala-D-Lac-O^{t}Bu (0.18 g, 0.33 mmol) led to **8** (0.11 g, 65%) as an orange solid. R_f=0.03 (DCM/methanol, 10:1). ^{1}H NMR (CD_3OD, 300 MHz) δ: 8.69 (d, 1H, H-6-NBD, J=8.8 Hz), 6.53 (d, 1H, H-5-NBD), 5.12, 4.66 (2m, 2H, CHα), 4.09 (d, 2H, CH_{2a}-Gly, J_{gem}=16.7 Hz), 4.02 (d, 2H, CH_{2b}-Gly), 3.72 (bs, 2H, CH_2NH-NBD), 2.49 (t, 2H, CH_2C(O)-NBD, J=7.3 Hz), 1.98, 1.89, 1.66 (3m, 6H, CH_2-NBD), 1.63, 1.62 (2d, 6H, $CH_3\beta$, J=7.4, 7.1 Hz). ES-MS (m/z)=517.45 (100%, $[M+Na]^+$), 495.19 (25%, $[M+H]^+$). HPLC (Rt=18.98 min, 94% purity).

4.2.3. Ds-Gly-D-Ala-D-Ala-OH (9). To a solution of **4** (0.27 g, 1 mmol) in 3% $NaHCO_3$, (10 mL), a solution of dansyl chloride (0.30 mL, 1.1 mmol) in aceton (10 mL) was added. After stirring the fluorescent solution in the absence of light overnight, the reaction mixture was evaporated to dryness in vacuo and the residue was dissolved in EtOAc (10 mL). The solution was washed with H_2O, dried (Na_2SO_4) and the solvent was evaporated. The crude product was purified by column chromatography using a gradient of DCM/methanol from 50:1 to 30:1 to give Ds-Gly-D-Ala-D-Ala-O^{t}Bu (0.41 g, 82%) as a light-green solid. R_f=0.48 (DCM/methanol, 9:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.51, 8.30 (2d, 2H, Ar, J=8.5 Hz), 8.21 (d, 1H, Ar, J=7.4 Hz), 7.50, 7.47 (2d, 2H, Ar, J=16.0 Hz), 7.36, 7.26 (2d, 2H, NH(CO), J=7.7 Hz), 7.13 (d, 1H, Ar), 6.92 (bs, 1H, $NHSO_2$), 4.52, 4.36 (2m, 2H, CHα), 3.61 (s, 2H, CH_2-Gly), 2.85 (s, 6H, $N(CH_3)_2$), 1.44 (s, 9H, $C(CH_3)_3$), 1.30, 1.13 (2d, 6H, $CH_3\beta$, J=7.0 Hz). ES-MS (m/z)=529.35 (16%, $[M+Na]^+$), 451.35 (100%, $[M-^tBu+Na]^+$).

After the cleavage of the ^{t}Bu ester of Ds-Gly-D-Ala-D-Ala-O^{t}Bu (0.17 g, 0.33 mmol), the reaction mixture was evaporated in vacuo and $KHSO_4$ (1 M), was added. The pH of the aqueous layer was adjusted to 3–4 by addition of NaOH (1 N) and extracted with EtOAc (70 mL) three times. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated to afford **9** (0.11 g, 65%) as a light-green solid. R_f=0.52 (methanol/chloroform/NH_4OH, 60:45:20). ^{1}H NMR (CD_3OD, 300 MHz) δ: 8.57, 8.34 (2d, 2H, Ar, J=8.5, 8.8 Hz), 8.21 (d, 1H, Ar, J=7.4 Hz), 7.54–7.63 (m, 2H, Ar), 7.27 (d, 1H, Ar, J=7.7 Hz), 4.93–4.22 (m, 2H, CHα), 3.54 (s, 2H, CH_2-Gly), 2.86 (s, 6H, $N(CH_3)_2$), 1.37, 1.17 (2d, 6H, $CH_3\beta$, J=7.4, 7.1 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 177.3 (COOH), 174.1, 170.7 (2NH(CO)), 153.3, 135.98 (Ar-*C*), 131.6 (Ar-*H*), 131.2, 130.9 (Ar-*C*), 130.6, 129.6, 124.3, 120.2, 116.7 (Ar-*H*), 50.3 (Cα-Ala), 46.4 (Cα-Gly), 45.8 ($N(CH_3)_2$), 18.0, 17.8 ($CH_3\beta$). ES-MS (m/z)=451.35 (100%, $[M+H]^+$). HPLC (Rt=15.93 min, 99% purity).

4.2.4. Ds-Gly-D-Ala-D-Lac-OH (10). The same procedure as was used for **9** was followed for the preparation of **10**. **5** (0.14 g, 0.5 mmol) gave Ds-Gly-D-Ala-D-Lac-O^{t}Bu as a light-green solid. Cleavage of the ^{t}Bu ester of Ds-Gly-D-Ala-D-Lac-O^{t}Bu led to **10** (0.17 g, 75% over 2 steps) as a light-green solid. R_f=0.63 (methanol/chloroform/NH_4OH, 60:45:20). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.52 (2d, 2H, Ar, J=8.5 Hz), 8.28 (d, 1H, Ar), 8.20 (1d, 1H, Ar, J=7.1 Hz), 7.74–7.56 (m, 2H, Ar), 7.19 (1d, 1H, Ar, J=7.7 Hz), 7.13 (d, 1H, NH(CO), J=7.4 Hz), 6.49 (bt, 1H, $NHSO_2$), 5.92 (bs, 1H, COOH), 5.01 (1m, 1H, CHα-Lac), 4.45 (1m, 1H, CHα-Ala), 3.59 (bs, 2H, CH_2-Gly), 2.88 (s, 6H, $N(CH_3)_2$), 1.48, 1.21 (2d, 6H, $CH_3\beta$, J=7.1, 7.4 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 173.6 (COOH), 172.2, 169.0 (2NH(CO)), 151.4, 133.6 (Ar-*C*), 130.7, 130.0 (Ar-*H*), 129.7, 129.4 (Ar-*C*), 128.8, 123.4, 118.8, 115.2 (Ar-*H*), 69.6 (Cα-Lac), 48.0 (Cα-Ala), 45.6 (Cα-Gly), 45.4 ($N(CH_3)_2$), 17.0, 16.6 ($CH_3\beta$). ES-MS (m/z)=452.3 (100%, $[M+Na]^+$). HPLC (Rt=15.90 min, 99% purity).

4.2.5. DR-C(O)–$(CH_2)_2$–C(O)-Gly-D-Ala-D-Ala-OH (11). To solution of **4** (0.27 g, 1 mmol) in DCM (2 mL), succinic anhydride (0.1 g, 1 mmol) and TEA (0.15 mL, 1.1 mmol) were added. After stirring the mixture at rt for 2 h, the reaction mixture was evaporated in vacuo and the residue was dissolved in butanol (3 mL). The solution was washed with $KHSO_4$ (1 M), H_2O, and brine. After drying (Na_2SO_4) and evaporation of the solvent, HOOC–$(CH_2)_2$–C(O)-Gly-D-Ala-D-Ala-O^{t}Bu (0.32 g, 87%) was obtained as a white

solid. R_f=0.23 (DCM/methanol, 10:1). ^{1}H NMR (DMSO, 300 MHz) δ: 12.16 (bs, 1H, COOH), 8.16 (d, 2H, 2 NH(CO), J=6.6 Hz), 7.94 (d, 1H, NH(CO), J=7.7 Hz), 4.30, 4.07 (2m, 2H, CHα), 3.66 (d, 2H, CH_2-Gly, J=5.7 Hz,), 2.41–2.35 (m, 4H, CH_2), 1.36 (s, 9H, $C(CH_3)_3$), 1.23, 1.19 (2d, 6H, $CH_3\beta$, J=7.4, 7.1 Hz). ^{13}C NMR (DMSO, 75 MHz) δ: 174.1, 172.1, 171.8, 171.8, 168.6 (COOH, COO, NH(CO)), 80.5 ($C(CH_3)_3$), 48.5, 47.78 (Cα-Ala), 42.3 (Cα-Gly), 30.0, 29.3 (CH_2), 27.8 ($C(CH_3)_3$), 18.5, 17.0 ($CH_3\beta$). ES-MS (m/z)=396.25 (100%, $[M+Na]^+$), 374.25 (7%, $[M+H]^+$), 318.10 (18%, $[M-{}^t\mathrm{Bu}+H]^+$).

HOOC–$(CH_2)_2$–C(O)-Gly-D-Ala-D-Ala-O^{t}Bu (0.31 g, 0.83 mmol), Disperse red-NH_2[16] (0.26 g, 0.83 mmol), BOP (0.37 g, 0.83 mmol) and DiPEA (0.29 mL, 1.66 mmol) were dissolved in DCM (50 mL). After stirring the dark red solution at rt overnight, the solvent was evaporated in vacuo and the residue was dissolved in EtOAc (10 mL). This solution was washed with $KHSO_4$ (1 M), H_2O, and brine. After drying (Na_2SO_4) and evaporating the solvent, the crude product was purified by column chromatography using a gradient of DCM/methanol from 50:1 through 30:1 to 10:1 and DR-C(O)–$(CH_2)_2$–C(O)-Gly-D-Ala-D-Ala-O^{t}Bu (0.28 g, 51%) was obtained as a dark red solid. R_f=0.58 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD, 300 MHz) δ: 8.27, 7.87 (2d, 4H, Ar, J=9.1 Hz), 7.84 (d, 2H, Ar, J=9.9 Hz), 7.39 (bs, 2H, 2 NH(CO)), 7.09 (d, 1H, NH(CO), J=7.4 Hz), 6.92 (bs, 1H, NH(CO)), 6.75 (d, 2H, Ar, J=9.3 Hz), 4.42, 4.32 (2m, 2H, CHα), 3.95, 3.76 (d, 2H, CH_2-Gly, J=16.8 Hz,), 3.51–3.38 (m, 6H, CH_2-N), 2.48 (s, 4H, CH_2–C(O)), 1.40 (s, 9H, $C(CH_3)_3$), 1.35, 1.31 (2d, 6H, $CH_3\beta$, J=7.1 Hz), 1.21 (t, 3H, CH_3, J=7.1 Hz). ES-MS (m/z)=691.50 (70%, $[M+Na]^+$), 669.50 (100%, $[M+H]^+$).

Cleavage of the ^{t}Bu ester of DR-C(O)–$(CH_2)_2$–C(O)-Gly-D-Ala-D-Ala-O^{t}Bu (0.20, 0.3 mmol), as was described above except for the use of dioxane instead of DCM, gave **11** (0.14 g, 75%) as a dark red solid. R_f=0.05 (DCM/methanol, 10:1). ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD, 300 MHz) δ: 8.06, 7.64 (2d, 4H, Ar, J=9.1 Hz), 7.62 (d, 2H, Ar, J=9.3 Hz), 6.57 (d, 2H, Ar, J=9.1 Hz), 4.06, 3.90 (2m, 2H, CHα), 3.66 (s, 2H, CH_2-Gly, J=16.8 Hz,), 3.26–3.10 (m, 6H, CH_2-N), 2.35–2.19 (m, 4H, $CH_2C(O)$), 1.16, 1.09 (2d, 6H, $CH_3\beta$, J=7.1 Hz), 0.97 (t, 3H, CH_3, J=7.1 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ: 179.3 (COOH), 175.5, 174.9, 173.7, 171.7 (COO, NH(CO)), 158.1, 153.0, 148.5, 144.7 (Ar-*C*), 127.2, 125.6, 123.4, 112.5 (Ar-*H*), 51.8, 50.6 (Cα-Ala), 46.3, 43.6, 38.1, 32.0, 31.9, 30.6 (CH_2N, $CH_2C(O)$, Cα-Gly), 19.3, 17.9 ($CH_3\beta$), 12.5 (CH_3). ES-MS (m/z)=635.40 (15%, $[M+Na]^+$), 613.45 (100%, $[M+H]^+$). HPLC (Rt=21.70 min, 99% purity).

4.3. General procedure for coupling Fmoc amino acids, $(Boc)_2$-CTV-OH (13) or $(Fmoc)_2$-CTV-OH (14) on the solid phase

The resin (1 equiv) was swollen in NMP (2 min) and, after draining the solvent, BOP (4 equiv), the desired Fmoc amino acid (4 equiv) and NMP (15 mL/mmol) were added. The mixture was shaken until complete dissolution and then DiPEA (8 equiv) was added. For coupling of **13** or **14**, 2 equiv of **13** or **14**, 2 equiv of BOP, and 4 equiv of DiPEA were used and the reaction was shaken at rt overnight. After 3 h of shaking, the resin was washed with NMP (5×15 mL/mmol, each 2 min) and DCM (5×15 mL/mmol, each 2 min). Negative Kaiser and BPB tests indicated completion of the coupling. The loading of the resin was determined by Fmoc-spectrophotometric quantification, after drying the resin under vacuum overnight.

4.4. General procedure for N$^\alpha$-Fmoc deprotection

The N$^\alpha$-Fmoc-protected resin was swollen in NMP (2 min) and, after draining the solvent, the resin was shaken with 20% piperidine in NMP (3×20 mL per mmol, each 10 min). The resin was washed with NMP (5×10 mL per mmol, each 2 min) and DCM (5×10 mL per mmol, each 2 min). Positive Kaiser and BPB tests indicated Fmoc-deprotection.

4.4.1. Loading of Argogel®-NH_2 or Argogel®-Rink-NH_2 (16a, 16b). Fmoc-L-Ala-OH·H_2O (1.05 g, 3.2 mmol) was coupled to Argogel®-NH_2 resin (**a**) (2 g, 0.8 mmol) or to Fmoc-deprotected Argogel®-Rink-NH_2 (**b**) (2.42 g, 0.8 mmol). Then the N$^\alpha$-Fmoc group was cleaved, following the general procedure and coupling of Fmoc-L-Ala-OH·H_2O was repeated twice. Prior to the last N$^\alpha$-Fmoc deprotection step, the loading was determined to be 0.31 and 0.25 mmol g^{-1}, respectively. After N$^\alpha$-Fmoc deprotection, **12a** was coupled to **13** to give **15a**, and **12b** was coupled to **14** to give **15b**. Treatment of **15a** with 50% TFA in DCM, (7 mL) for 45 min and additional washing of the resin with NMP (3×7 mL, each 2 min), 25% DiPEA in NMP (4×7 mL, each 2 min), NMP (5×7 mL, each 2 min), and finally DCM (5×7 mL, each 2 min) led to resin **16a**. Fmoc-deprotection of **15b** using the general procedure gave resin **16b**.

4.4.2. Resynthesis of receptors 19a and 20a on Argogel™-NH_2 resin and 17b and 18b on the Argogel®-Rink resin. Resins **16a** and **16b** (each containing 0.051 mmol of amino groups) were dried overnight under vacuum and were separately swollen in NMP (for 2 min). After draining the solvent, three subsequent coupling/N$^\alpha$-Fmoc deprotection cycles were carried out of Fmoc-L-Lys(Boc)-OH, Fmoc-L-Leu-OH and Fmoc-L-Lys(Boc)-OH following the general procedures to give **17a** and **17b**. The same procedure was followed for the synthesis of **18a** and **18b**, except for the use of Fmoc-L-Glu(O^{t}Bu)-OH, Fmoc-L-Ala-OH, and Fmoc-L-Ser(^{t}Bu)-OH as amino acids. The Boc groups from **17a** and **18a** were cleaved using 95% TFA in H_2O, 95:5. The resin was washed with NMP (3×1.5 mL, each 2 min), 25% DiPEA in NMP: (4×1.5 mL, each 2 min), NMP (5×1.5 mL, each 2 min), DCM (5×1.5 mL, each 2 min), dioxane (4×1.5 mL, each 2 min), dioxane/H_2O, 1:1 (4×1.5 mL, each 2 min), H_2O (4×1.5 mL, each 2 min), and Et_2O (5×1.5 mL, each 2 min) to give the receptors **19a** and **20a**, respectively.

4.4.3. Deprotection of the acid labile side-chains and cleavage of the receptors 17b and 18b from the Argogel®-Rink resin. Receptors **17b** and **18b** on the Argogel®-Rink resin were swollen in DCM (2×2 min) and the solvent was drained. After addition of 95% TFA in H_2O (20 mL/mmol), resins were shaken at rt for 4 h, removed by

filtering and washed with TFA/H_2O, 95:5. The filtrates were combined and Et_2O was added to give suspensions of the cleaved synthetic receptors. After centrifugation (3500 ppm, 5 min) of the suspensions, the volatiles were removed and the white solids were dissolved in H_2O followed by lyophilization to give trifluoroacetate salt of **21** (86 mg, 76%); HPLC: Rt = 17.80 min, 94% purity, ES-MS (*m/z*) = 766.95 (100%, $[M+2H]^{2+}$), 511.50 (100%, $[M+3H]^{3+}$), 383.75 (58%, $[M+4H]^{4+}$), and trifluoroacetate salt of **22** (56 mg, 69%); HPLC: Rt = 17.68 min, 94% purity; ES-MS (*m/z*) = 1390.00 (25%, $[M+Na]^{+}$), 1368.30 (100%, $[M+H]^{+}$), 684.60 (60%, $[M+2H]^{2+}$), respectively.

4.5. Procedure for liberation of the amine from the TFA-salt

A solution of trifluoroacetate salt of **21** (6.6 μmol) in acetonitrile/H_2O (1:1) (1.2 mL) was added to Amberlyst A21-ion exchange resin (264 μmol) and the resin was shaken for 45 min. After filtration, the solution was lyophilized to give quantitatively **21**. In case of the trifluoroacetate salt of **22**, Dowex 50×8 (H^+ form) ion exchange resin (1 g) was used. The resin was shaken for 4 h and the solvent was drained. 25% NH_3 in H_2O was added to the resin and shaking was continued for 1 h. After filtration the solution was lyophilised to give **22**.

4.6. Screening of library 1{1-13, 1-13, 1-13} with D-Ala-D-Ala and D-Ala-D-Lac containing ligands (6–11)

A 1 mg mL^{-1} suspension of **6**, a 50 mM solution of **7**–**10**, and a 5 mM solution of **11** in phosphate buffer (0.1 N, pH = 7.0) (1.3 mL) were added to different portions of library **1**{1-13, 1-13, 1-13} (14 mg, ~11.000 beads, ~4 copies/receptor) and incubated at 20 °C for 72 h. The resin was drained and was washed with phosphate buffer (0.1 N, pH = 7.0) (5×1.3 mL, each 2 min). Next, the resin was poured into a petri dish and spread into a monolayer. The beads were viewed under a fluorescence microscope. By use of a long needle, most fluorescent or colored beads were isolated (~100 beads). The fluorescence of the preselected beads was reevaluated using the overexposure mode of the Leica DC-100 digital camera system and image analysis to estimate the relative fluorescence intensities semi-quantitatively. Reevaluation of the red colored beads in the case of **11** was judged by eye. Only nine to ten best fluorescent or colored beads were selected and analysed by independent Edman degradation together with a non-fluorescent bead from each screening.

4.7. Binding affinity for with D-Ala-D-Ala containing ligands (6, 7, 9 and 11 by 19a and 20a)

A 50 μM solution of **7** or **9** (1.45 mL) or a 5 μM solution of **11**, or a 1 mg mL^{-1} (433 μL) suspension of **6** in phosphate buffer (0.1 N, pH = 7.0) was added to the resynthesized receptors **19a** or **20a** (0.6 mg, ~480 beads) and incubated at 20 °C for 72 h. The resins were drained and washed with phosphate buffer (0.1 N, pH = 7.0) (5×1.3 mL, each 2 min). The resin was poured into a petri dish and spread into a monolayer. The beads were viewed under a fluorescence microscope.

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